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Short communication

Spatial response of *Medicago truncatula* plants to drought and spider mite attack

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ABSTRACT

Plant response to imposition of biotic and abiotic stresses by inducing their defense mechanisms, with the production of reactive oxygen species (ROS) representing a major defense response. The present work examined the simultaneous impact of two key stress factors, drought and spider mite attack (*Tetranychus urticae*) in *Medicago truncatula* plants. Hydrogen peroxide (H_2O_2), lipid peroxidation (MDA content) and proline content in well-watered and drought-stressed leaves infested by spider mites along with neighboring leaves were examined in order to investigate the local and systemic effect of the two stresses on the antioxidant and osmoprotective response. High levels of lipid peroxidation were recorded in plants under drought stress and plants under combined drought stress and spider mite feeding compared with control plants. Hydrogen peroxide biosynthesis was significantly induced in plants under drought and spider mite attack, with highest levels detected in the feeding leaf (local response). Proline was accumulated in drought stressed-plants, with the highest levels observed in plants exposed to a combination of drought stress and mite feeding. RT-qPCR expression analysis of key genes implicated in ROS metabolism (*PAO, DAO, AOX, CuZnSOD, FeSOD, MnSOD*) and proline biosynthesis (*P5CR, P5CS*) pointed to different patterns of regulation between abiotic and biotic stress, as well as their combination. Exposure of plants to both drought stress and attack by spider mites mainly affected the local antioxidant and osmoprotective response of *Medicago truncatula*, highlighting the relative significance of drought-induced phenomena in combined drought/mite infestation stress responses.

1. Introduction

Climate change is expected to increase the frequency of plant exposure to multiple abiotic and biotic stresses. The simultaneous occurrence of different stresses causes detrimental effects in plant productivity which lead to significant losses in modern agriculture. According to the Intergovernmental Panel on Climate Changes, global surface temperature is projected to increase about 2 °C, while mean precipitation is expected to decrease by the end of 21st century, increasing the drought periods especially in semiarid zones (Field et al., 2014). Drought is one of the most devastating abiotic stress factors limiting plant productivity and yield production worldwide (Filippou et al., 2011; Mhadhbi et al., 2011; Krasensky and Jonak, 2012). Herbivore attack is another important factor of limiting food production since it is estimated to cause 10–20% losses in major crops (Ferry et al., 2004). It is well-documented that drought conditions advocate the potentiality of herbivore outbreaks. However, the relation between drought stress and herbivore infestation is closely dependent on the timing and the severity of drought stress, the type of plant and the type of feeding guild that the herbivores belong to (Huberty and Denno, 2004; Gutbrodt et al., 2011).

In many case plants respond to biotic and abiotic stresses by activating multiple signal transduction pathways in order to enhance plant stress tolerance, while in other cases stress conditions potentially lead to plant cell death. In general, drought conditions induce multiple physiological, biochemical and molecular responses in plants such as stomatal closure, repression of photosynthesis, accumulation of compatible osmolytes (i.e. sugars alcohols, proline), induction of antioxidant mechanism and regulation of stress responsive genes with diverse function (Jakab et al., 2005; Shinozaki and Yamaguchi-Shinozaki, 2007; Chaves et al., 2009; Miller et al., 2010). Drought-stressed plants are more susceptible to attracting herbivores since they have higher nutritional value due to increased amount of free sugars and free amino acids (Hummel et al., 2010; Showler, 2013). Herbivore feeding induces several plant responses such as volatile emission and accumulation of phenolic compounds that are regulated by a complex network of

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hormones with jasmonic acid and salicylic acid being proposed as having an indispensable role (Ament et al., 2004; Van Poecke and Dicke, 2004; Leitner et al., 2005; Maffei et al., 2007). A common regulation of both drought stress and herbivore attack is the induction of changes in ROS equilibrium by triggering the ROS production leading to an oxidative burst (Bhattacharjee, 2005). Hormonal regulation has also been implicated in the response (Alba et al., 2015; Peleg and Blumwald, 2011).

The majority of the studies performed normally focus on understanding plant responses to individual stress exposure. However, this is far from reality, since multiple stresses occur simultaneously under field conditions, affecting plant growth and survival. Additionally, the response of plants to a combination of stresses is unique and it cannot be predicted by studying the responses of plants to each stress separately (Suzuki et al., 2014). It is therefore of crucial importance to study the combinatory effect of stresses to plants. The present study attempts to provide insights into the combined effects of drought and *Tetranychus urticae* attack to the cellular status as well as to antioxidant and osmoprotectant responses in the model legume plant *Medicago truncatula*. Emphasis was also given to unravel plant responses after mite attack at a local and systemic level.

2. Material and methods

2.1. Plant material and mite rearing

Medicago truncatula ecotype Jemalong A17 plants were grown until the mature stage (40 d old). Seeds were sown in sterile perlite:pot soil (1:3) pots (volume: 0.52 L) and stratified at 4 °C for 4 d. Plants were grown in a growth room at 22/16 °C day/night temperatures, and 60–70% relative humidity (RH), 16:8 L:D photoperiod with a photosynthetic photon flux density of ~100 μmol PAR m^{-2} s^{-1}. Drought was imposed by withholding watering for 9 d (Filippou et al., 2011). *Tetranychus urticae* mites were collected from cucumber plants (*Cucumis sativus*) from a commercial greenhouse in Pareklissia, Limassol, Cyprus, and were reared on bean plants (*Phaseolus vulgaris*) for two weeks until use in the experiments. Eight adult *T. urticae* females were positioned at the top leaflet of the ternate compound leaf of either well-watered or drought-stressed plants. Lanoline was placed with the aid of fine brush around the top leaflet to prevent the mites from moving and settling on adjacent leaflets. Mites were allowed to settle for 24 h prior to the experiments (Fig. S1). Twenty four hours later, the mites were removed from the top leaflet and both the top leaflet and the two neighboring leaflets were separately harvested to study the local and systemic effect of phytophagy. Compound leaves harvested from control and drought stressed-plants not exposed to mites were also harvested. Analyses were carried out using a minimum of three independent biological replicates in each experiment. Each replicate consisted of pooled leaves from six independent plants.

2.2. Lipid peroxidation, hydrogen peroxide and proline quantification

Lipid peroxidation was quantified by measuring of malondialdehyde (MDA) content resulting from the thiobarbituric acid (TBA) reaction (Minotti and Aust, 1987), using an extinction coefficient of 155 mM^{-1} cm^{-1}. Hydrogen peroxide was determined following the KI method, as described by Velikova et al. (2000). Free proline levels were measured using the ninhydrin reaction (Bates et al., 1973). H_2O_2 and proline concentrations were estimated by performing standard curves.

2.3. RT-qPCR analysis

Total RNA was extracted from leaves using TRIzol (TRI reagent; Sigma-Aldrich, USA) according to the manufacturer's instructions, followed by DNase digestion (Cat. No. NU01a, HT Biotechnology LTD, England), in order to remove gDNA. The quantity and the quality of the RNA was analysed spectrophotometrically using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA) and the RNA integrity was checked by gel electrophoresis. For cDNA synthesis, 1 μg of total RNA was reversed transcribed using Primescript 1st Strand Synthesis Kit (Takara, Japan), according to the manufacturer's protocol. Subsequently, real-time RT-qPCR was performed using Biorad IQ5 (Biorad, USA) with SYBR Green I master (KAPA SYBRR FAST qPCR Kit, Kapa-Biosystems, USA), according to the manufacturer's instructions. Primer sequences of the products are listed in Supplementary Table S1. Relative quantification of gene expression and statistical analysis of all RT-qPCR data (pairwise fixed reallocation randomization test) were performed using the REST software according to Pfaffl et al. (2002). The *ACTIN11* gene was used as a housekeeping reference gene (Mhadhbi et al., 2011).

2.4. Statistical analyses

Statistical analyses was performed using the software package SPSS version 21.0 (SPSS, Chicago, USA). The comparison of averages of each treatment was based on the analysis of variance (one-way ANOVA) according to Duncan's multiple range test at a significance level of 5% ($P \leq 0.05$). In all analyses, test of normality was carried out and the significance on the Kolmogorov-Smirnov method (Massey Jr, 1951) was checked.

3. Results

3.1. ROS production in drought-stressed and mite-infested plants at local and systemic level

Oxidative damage was initially investigated in leaves harvested from control and drought stressed-plants, as well as mite-infested leaflets (local effect) and the neighboring leaflets (systemic effect) of well-watered and drought stressed-plants. Oxidative damage levels were estimated by measuring the levels of lipid peroxidation (MDA content) and H_2O_2, which is a representative reactive oxygen species (ROS) that is relatively stable compared other ROS (Sharma et al., 2012). MDA content was higher in plants under drought stress conditions and plants under combined drought stress and spider mite feeding compared with control plants (Fig. 1A). However, spider mite feeding did not cause any further significant increase in lipid peroxidation levels (Fig. 1A). On the other hand, the production of H_2O_2 followed a different pattern compared with MDA content. H_2O_2 was significantly induced in plants under drought and spider mite attack both locally and systemically, with the highest levels detected on the feeding leaflet (local response) of drought-stressed plants (Fig. 1B). Gene expression analysis was performed by studying the transcript levels of enzymes involved in ROS metabolism. Gene expression of enzymes implicated in H_2O_2 production (*PAO*, *DAO*, *CuZnSOD*, *FeSOD, MnSOD*) was in general induced by both drought stress and mite attack (Fig. 1C). PAO (polyamine oxidase) and DAO (diamine oxidase) are involved in polyamine scavenging pathway, through which H_2O_2 is produced. *PAO* transcript levels were induced in drought-stressed plants as well as in mite feeding and neighboring leaflets of well-watered plants, while a suppression was observed in mite feeding leaflets (local effect) of drought-stressed plants (Fig. 1C). *DAO* expression was significantly induced by mite infestation in the feeding leaflets independently of the presence or absence of drought stress (Fig. 1C). Superoxide dismutase (SOD) catalyzes the oxidation of superoxide (O_2^-) to H_2O_2. The expression profile of three SOD isoforms (*CuZnSOD*, *FeSOD, MnSOD*) was studied and an up-regulation of *CuZnSOD* and *FeSOD* was observed in drought-stressed plants. *CuZnSOD* was highly induced both in drought-stressed and mite-infested plants, with highest expression levels recorded in the neighboring leaflets of mite feeding and well-watered plants. *FeSOD* transcript levels were lower than those of *CuZuSOD* in mite-infested leaves (Fig. 1C). Contrarily, the expression of *MnSOD* which is located at

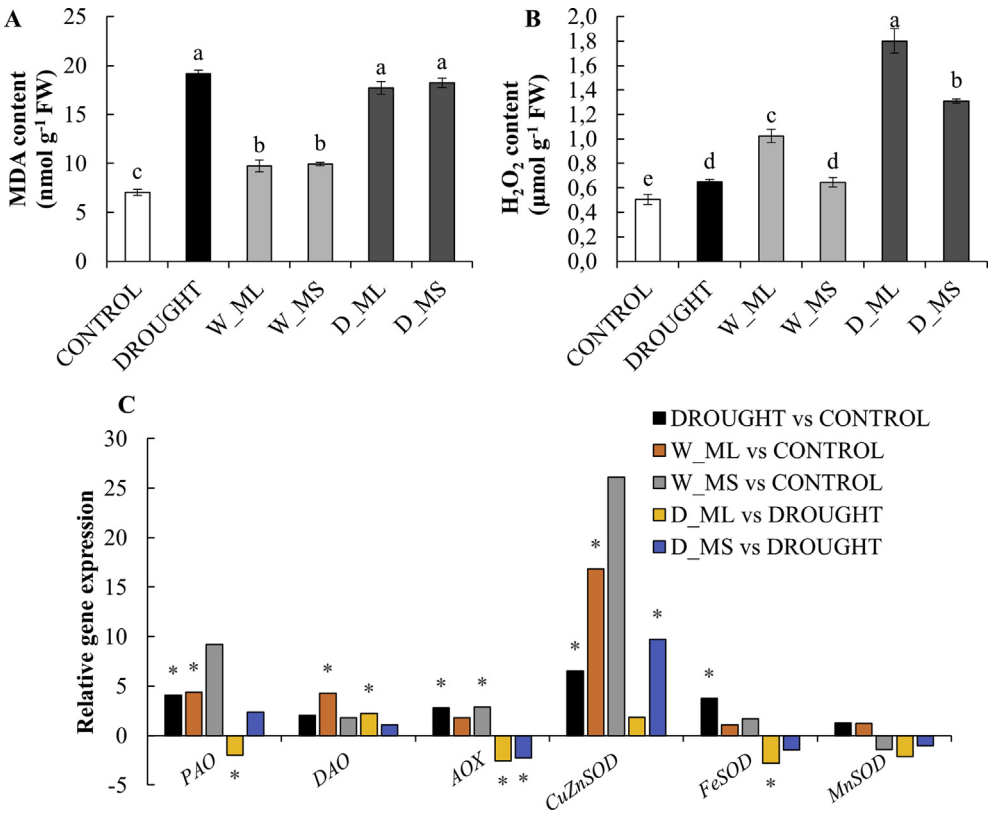

Fig. 1. Effect of drought and mite feeding in ROS metabolism in leaves of mature *M. truncatula* plants. (A) Lipid peroxidation (MDA) and (B) hydrogen peroxide (H_2O_2) contents and (C) gene expression profile of enzymes implicated in hydrogen peroxide metabolism (*PAO*, *DAO*, *AOX*, *CuZnSOD*, *FeSOD*, *MnSOD*). W_ML, local effect on mite feeding leaflets in watered plants. W_MS, systemic effect on site leaflets in watered plants; D_ML, local effect on mite feeding leaflets in drought stressed-plants; W_MS, systemic effect on site leaflets in drought stressed-plants. Data donated with different letters and * represent significant differences ($P < 0.05$). Error bars indicate standard error.

mitochondria was not affected by any of the treatments. On the other hand, alternative oxidase (*AOX*) that lower ROS production in mitochondria, by reducing the O_2^- formation, was found to be suppressed under the combination of drought and mite attack conditions, while it was induced in individually applied stresses (drought-stressed or mite-infested plants; Fig. 1C).

3.2. Effect of drought stress and mite attack in proline biosynthesis

Free proline content and gene expression levels of *P5CS* and *P5CR*, which are the key regulatory enzymes in the proline biosynthetic pathway, were measured in drought-stressed and mite-infested *M. truncatula* plants (Fig. 2A). Proline content was increased in drought stressed-plants, with the highest increase recorded in plants under combined abiotic + biotic stress (Fig. 2A). A slight decrease in proline content was observed in the neighboring leaflet of mite feeding and well-watered plants (Fig. 2B). However, mite feeding in well-watered plants did not affect proline biosynthetic enzyme gene expression. *P5CS* and *P5CR* gene expression was found to be significantly induced in drought stressed-plants (Fig. 2B). Mite attack systemically induced *P5CS* expression in the neighboring leaflets of drought-stressed plants, whereas *P5CR* was suppressed (Fig. 2B). Moreover, a significant induction of *P5CR* was observed in mite feeding leaflets (locally) of drought-stressed plants (Fig. 2B).

4. Discussion

Plants are exposed a plethora of abiotic and biotic stress factors that can occur simultaneously, resulting in a co-activation of different defense response pathways. In many cases, the exposure of a plant to a stress factor increases the probability of a second stress to occur. A well-documented example in nature is the exposure of plants to the combination of drought and herbivore attack. Drought-stressed plants induced the production of many nutritional molecules such as sugars and amino acid as a part of their osmoprotective response, while other defense pathways are also induced (Leitner et al., 2005; Maffei et al., 2007; Ximénez-Embún et al., 2017). The balance between the nutritional status and the chemical defense determined the potentiality of a mite attack to occur. Several studies have been performed showing that drought stressed plants may be more prone to *Tetranichus sp.* infestation (Leitner et al., 2005; Sivritepe et al., 2009; Ximénez-Embún et al., 2016). In this study, the local and systemic effects of *T. urticae* feeding under drought-stressed and well-watered growth conditions were investigated. More specifically, emphasis was given in understating the antioxidant and osmoprotectant responses of plants to mite-infested and drought-stressed plants in individual application and in combination.

Both drought stress and mite attack increase the production of H_2O_2, which enhances signaling processes at low concentrations while leading to oxidative damage at high levels (Gill and Tuteja, 2010). Several reports have demonstrated an increase in cellular damage after the exposure to individually applied drought stress and mite attack (Walling, 2000; Bhattacharjee, 2005; Sivritepe et al., 2009; Filippou et al., 2011). According to the current findings, mite feeding significantly increased H_2O_2 content at local and systemic level, 24 h after infestation commenced. Although H_2O_2 production was more pronounced in feeding leaflets, mite attack caused significant increase in MDA content at both local and systemic level. Interestingly, plants exposed to combined stress of mite feeding and drought demonstrated enhanced H_2O_2 production, suggesting a synergistic effect of both stresses in regulating ROS production. However, plant exposed to the specific combination of stresses showed similar levels of lipid peroxidation as drought-stressed plants.

Plants respond to ROS accumulation by activating the antioxidant machinery through the regulation of genes implicated in ROS detoxification (Mittapalli et al., 2007; Miller et al., 2010). Differential expression profile of ROS metabolism genes was observed after the application of individual stresses and their combination. A significant induction of almost all the examined ROS-related genes was observed in drought-stressed plants, in accordance with previous studies (Ahmad

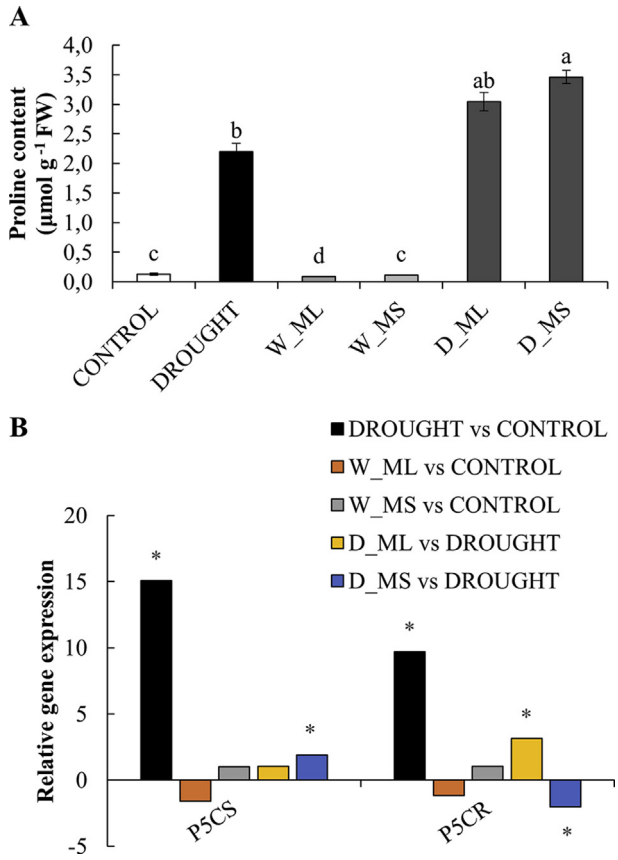

Fig. 2. Effect of drought and mite feeding in the production of the osmoprotective molecule proline in leaves of mature *M. truncatula* plants. (A) Proline content and (B) gene expression profile of enzymes implicated in proline biosynthesis (P5CS, Δ1-pyrroline-5-carboxylate synthetase; P5CR, Pyrroline-5-carboxylate reductase). W_ML, local effect on mite feeding leaflets in watered plants. W_MS, systemic effect on site leaflets in watered plants; D_ML, local effect on mite feeding leaflets in drought stressed-plants; W_MS, systemic effect on site leaflets in drought stressed-plants. Data donated with different letters and * represent significant differences ($P < 0.05$). Error bars indicate standard error.

et al., 2008; Filippou et al., 2011). Similarly, the mite-infested plants showed an induction in expression levels of almost all the studied genes at local and systemic level. It is well known that SODs constitute the first line enzymes of defense against ROS accumulation by scavenging O_2^- radicals (Alscher et al., 2002). Several SOD isoforms are located in different cell compartments in which O_2^- radicals are formed. Mite-infested plants revealed a pronounced induction of *CuZnSOD*, which is located in chloroplast and cytosol, highlighting the importance of this enzyme in ROS detoxification. Contrarily, the expression of the mitochondrial *MnSOD* was not affected by both stresses, while the expression of *FeSOD* which occur in chloroplast was induced in drought-stressed plants and suppressed in mite-infested and drought-stressed plants. Interestingly, *MnSOD* and *AOX* which both control ROS production in mitochondria, showed distinct expression profile under both stresses. It is worth mentioning that mite attack in drought-stressed plants suppressed the expression of *PAO*, *AOX* and *FeSOD* genes which were contrarily induced in mite-infested and non-drought stressed plants. This provides a notion that the combination of abiotic + biotic stress potentially leads to antagonistic effect among the stressors (Mittler, 2006).

Beside the induction of antioxidant machinery, plants defend against stresses by accumulating osmolytes such as sugar alcohols, crucial amino acids (proline) and glycine-betaine (Ashraf and Foolad, 2007). Proline is an important osmoprotectant molecule which accumulates in drought-stressed plants and acts as protein-compatible hydrotrope and radical scavenger (Matysik et al., 2002). In the present study, proline content was found to be increased in drought-stressed plants as expected, while molecular support in its biosynthesis was obtained through the induction of transcript levels of the main enzymes implicated in proline biosynthesis (*P5CS* and *P5CR*). Although mite infestation did not affect proline content, the combination of drought and mite attack significantly increased proline content in higher levels compared with drought-stressed plants, suggesting a synergistic effect of both stresses, in accordance with previous reports (Sarmento et al., 2011; Alba et al., 2015; Ximénez-Embún et al., 2017).

In conclusion, this study identified the existence of an interaction between drought stress and mite attack in regulating H_2O_2 metabolism. Exposure of plants to both drought stress and attack by spider mites affected mainly the local oxidative response in *M. truncatula*. Interestingly, the antioxidant and osmoprotective responses of plants differed between solo stresses and their combination at the time point examined, highlighting the importance to study the effects of multiple stress factors which represent more realistic agricultural production scenaria. By understanding the complex mechanism through which plants respond to abiotic and biotic stress combinations, substantial information will be provided for developing plants that are capable to survive under such environments.

5. Contribution

VF conceived the experiments, helped with analysis and writing the manuscript. CA performed the experiments, analysis and writing. IF helped with drought stress treatment and analysis. KM helped with mite infestation and analysis. MS helped conceive the experiments and writing the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.plaphy.2018.08.018.

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